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Complete List of Authors:	Anty, Rodolphe; INSERM U 895, Equipe 8; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; University of Nice Sophia-Antipolis Iannelli, Antonio; INSERM U 895, Equipe 8; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; University of Nice Sophia-Antipolis Patouraux, Stéphanie; INSERM U 895, Equipe 8; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; University of Nice Sophia-Antipolis Bonnafous, Stephanie; INSERM U 895, Equipe 8; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; University of Nice Sophia-Antipolis Lavallard, Vanessa; INSERM U895, Equipe 8; University of Nice-Sophia Antipolis Senni-Buratti, Massimo; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif Ben Amor, Imed; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif Myx-Staccini, Aline; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif Saint-Paul, Marie-Christine; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif Berthier, Frédéric; Hôpital Princesse Grace, Service d'information Médicale Huet, Pierre-Michel; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif Le Marchand-Brustel, Yannick; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; INSERM U 895, Equipe 8; University of Nice-Sophia Antipolis Gugenheim, Jean; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; INSERM U 895, Equipe 8; University of Nice-Sophia Antipolis Gual, Philippe; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; INSERM U 895, Equipe 8; University of Nice-

	Sophia Antipolis Tran, Albert; Hôpital Archet 2, Centre Hospitalier Universitaire de Nice, Pôle Digestif; INSERM U 895, Equipe 8; University of Nice-Sophia Antipolis

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Rodolphe Anty[1,2,3], Antonio Iannelli[1,2,3], Stéphanie Patouraux[1,2,4], Stéphanie Bonnafous[1,2,3], Vanessa Lavallard[1,2,3], Massimo Senni-Buratti[2], Imed Ben Amor[2], Aline Staccini-Myx[2], Marie-Christine Saint-Paul[1,4], Frédéric Berthier[5], Pierre-Michel Huet[2], Yannick Le Marchand-Brustel[1,2,3], Jean Gugenheim[1,2,3], Philippe Gual[1,2,3], Albert Tran[1,2,3]

[1]Institut National de la Santé et de la Recherche Médicale (INSERM), U895, Team 8, "Hepatic Complications in Obesity", Nice, F-06204, Cedex 3, France

[2]Centre Hospitalier Universitaire of Nice, Digestive Center, Nice, F-06202, Cedex 3, France

[3]University of Nice-Sophia-Antipolis, Faculty of Medecine, Nice, F-06107, Cedex 2, France

[4]Centre Hospitalier Universitaire of Nice, Biological Center, Nice, F-06107, Nice, Cedex 2, France

[5]Centre Hospitalier Princesse Grace, Medical Information Department, Monaco, MC 98012, Monaco.

Correspondance :

Dr Rodolphe ANTY, Centre Hospitalier Universitaire of Nice, Pôle Référence Hépatite C, Hôpital de l'Archet 2, 151, Route Saint-Antoine de Ginestière. BP 3079, F, 06202 Nice, Cedex 3, France.

Mail: anty.r@chu-nice.fr, Phone: (33) 04 92 03 59 43 (from outside of France, don't dial the 0), Mobile Phone: (33) 06 67 48 53 65 (from outside of France, don't dial the 0)

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Keywords: fatty liver, Alanine Aminotransferase, Metabolic syndrome, Cytokeratin-18 fragment levels, scoring system

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ABSTRACT (200 words)

Background and aims: Non invasive approaches are useful to differentiate simple steatosis from non-alcoholic steatohepatitis (NASH) in obese and morbidly obese patients. The aim of this study was to develop a new scoring system to diagnose definitive NASH.

Methods: Preoperative clinical and biological data including serum caspase 3-generated cytokeratin-18 fragments (CK18) and peroperative liver biopsies were obtained from 464 morbidly obese patients who had undergone bariatric surgery. The cohort was divided into two groups: training group (n=310) and validation group (n=154). Definitive NASH was defined according to Kleiner's classification with a NAFLD Activity Score (NAS) ≥5.

Results: Alanine aminotransferase (ALT), CK18 fragments and the presence of metabolic syndrome (MS) were independent predictors for discriminating patients with NAS ≥5 in the training group. These three parameters were used to carry out a scoring system for the prediction of NAS ≥5. Whereas serum CK18 fragment alone had an Area under the Receiver Operating Characteristic (AUROC) curve = 0.74, AUROC curves of the scoring system were 0.88 and 0.83 in the training and in the validation group respectively.

Conclusion: A simple and non invasive composite model including MS, ALT and CK18 fragments was able to accurately predict NAS ≥5 in morbidly obese subjects.

MAIN TEXT

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of pathological liver abnormalities ranging from pure steatosis to non-alcoholic steatohepatitis (NASH), and finally to severe fibrosis and cirrhosis.[1] Classically, pure steatosis has a relatively good prognosis whereas steatohepatitis is associated with progression of liver fibrosis.[2, 3] Thus, differentiation of these two states is important. NAFLD is common in the context of morbid obesity and NASH can be present in 20–40% of these patients.[4, 5]

The mechanisms of NAFLD development are complex and poorly understood. Chronic low grade chronic inflammation, adipokines dysregulation and insulin resistance in the liver and visceral adipose tissue are the major contributing factors.[6, 7] NAFLD has been considered as the hepatic manifestation of so-called "metabolic syndrome"(MS), a syndrome highly related to morbid obesity with its well defined components: increased waist circumference, high blood pressure, high blood triglycerides, low blood high-density lipoprotein (HDL) cholesterol, and high blood glucose level.[5, 8-10] Hepatocyte apoptosis may also play an important role in the liver injury and disease progression in NAFLD.[11]

Liver biopsy is still considered the gold standard to assess hepatic pathology in most chronic liver diseases, including NAFLD, but it is an invasive technique. Moreover, liver biopsy is associated with sampling and inter-observer variability, which might explain the differences in prevalence of NASH in morbidly obese patients reported in previous studies.[4, 12, 13] In addition, the large number of patients with morbid obesity exposed to NAFLD renders systematic liver biopsy in all patients difficult. The availability of a non-invasive test would allow systematic diagnosis in this particularly predisposed subpopulation and would enable repeated assessment during the follow-up in a more feasible way than liver biopsy. Unfortunately, medical imaging techniques (ultrasound, computerized tomography and magnetic resonance imaging) fail to differentiate pure steatosis from NASH.[14] Moreover, echography in severely or morbidly obese patients is frequently of poor quality.

Several non-invasive scoring models using routine clinico-biological parameters have been proposed for the diagnosis of NASH, but most of them have not been thoroughly validated.[15-19]

Recently, apoptotic caspase-3 generated cytokeratin 18 (CK18) fragments in the blood were reported to predict histological NASH in cohorts of obese patients or in patients with insulin resistance.[20-23]

The aim of this study was to develop a scoring system to diagnose definitive NASH using clinical and biological data related to the metabolic syndrome and liver injury including caspase-3 generated CK18 fragment, in a large cohort of European morbidly obese patients.

Patients and Methods

Study population

Four-hundred and sixty four consecutive morbidly obese patients, referred for bariatric surgery, were included between January 2003 and April 2009. A division of the initial cohort in 2 groups was carried out in order to be able to have a training and a validation patient cohort. The patients were arbitrarily divided into two groups: (i) a training patient cohort consisting of 310 patients (43 men, 267 women, mean age 40.0±10.7 years) enrolled from January 2003 to April 2007; and (ii) a validation patient cohort consisting of 154 patients enrolled from May 2007 to April 2009.

The study protocol was performed according to French legislation regarding Ethic and Human Research and was approved by the local Ethics Committee (Huriet-Serusclat law, DGS 2003/0395). Written informed consent was obtained from all patients. All patients met the 1991 NIH Consensus Conference guidelines for gastrointestinal surgery for obesity.[24] All patients were negative for hepatitis B and C viral markers, autoantibodies indicative of autoimmune hepatitis, and had negligible alcohol consumption (<20 g/day). Alcohol abuse was also excluded by interviewing the patients' relatives. Patients with a history of inflammatory disease (including rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease), current infections, recent history of cancer (<5 years), and severe pulmonary or cardiac disease were not enrolled in the study. All patients underwent a Roux-en-Y divided gastric bypass and a surgical liver biopsy was obtained during surgery.

Preoperative assessment

Preoperative assessment included: history and physical examination; blood pressure determination; anthropometric investigations (weight, height, waist circumference); psychiatric and nutritional evaluation; blood samples were also obtained before surgery after overnight fasting for the determination of plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ glutamyl transferase (γGT), glucose, insulin, C-peptide, glycosylated hemoglobin, triglycerides, HDL cholesterol, low density lipoprotein (LDL) cholesterol, ferritin. ALT and AST levels were determined using the Roche® assay with pyridoxal phosphate on a Hitachi Modular® according to the International Federation of Clinical Chemistry and the Société Française de Biologie

Clinique recommendations. Glycosylated hemoglobin was obtained with the Dual kit A1c Biorad® on a Variant 2 Biorad® (Biorad, USA). Blood levels of apoptotic caspase-3 generated CK18 fragment levels were measured using ELISA kit M30-Apoptosense® (Peviva, Bromma, Sweden) according to the manufacturer's instructions. All patients also had a chest X ray, electrocardiogram, abdominal ultrasound and upper gastrointestinal endoscopy.

Insulin resistance was assessed by homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR is the product of fasting plasma insulin concentration (mIU/L) and glucose concentration (mmol/L) divided by 22.5.[25]

MS was diagnosed according to the International Diabetes Federation (IDF) as follows: central obesity defined by a increased waist circumference (≥80 cm in women and ≥94 cm in men) and any two of the following criteria: (i) triglycerides ≥1.7 mmol/L or treatment for hypertriglyceridemia; (ii) HDL-cholesterol <1.29 mmol/L in women and <1.03 mmol/L in men; (iii) systolic blood pressure (BP) ≥130 mmHg or diastolic BP ≥85 mmHg or treatment for hypertension; and (iv) fasting plasma glucose ≥5.6 mmol/L or previously diagnosed type-2 diabetes. (8) All the morbid obese patients of this study had a rise in the waist circumference. Type-2 diabetes was defined by two measurements of elevated fasting plasma glucose ≥7 mmol/L.

Pathological liver assessment

Hepatic wedges were obtained during bariatric surgery. Surgical liver biopsies were reviewed by two liver pathologists (MC-SP and SP) without knowledge of the clinical or biological characteristics of the patients. Routine haematoxylin-eosin-safran and sirius red staining were performed on all biopsies. All biopsies were graded according to the NAFLD Activity Score (NAS). This score classifies patients as simple steatosis (NAS≤2), borderline group (3≤NAS≤4) or definitive NASH (NAS≥5).[26] Patients were analyzed as with or without NAS ≥5.

Liver bridging fibrosis was assessed by sirius red staining and was classified into five stages as follows: absent, mild, moderate (incomplete septa), severe (with complete septa) and cirrhosis.

Statistical analysis

Statistical analysis was first performed on the training patient cohort (n=310) in three steps: (i) univariate analysis of clinical and biochemical parameters was carried out for patients with NAS <5 and

NAS ≥5. The most relevant parameters for classifying the patients were noted. Continuous data are described as means ± standard deviation (SD). Comparisons were done using the Chi^2 test or Fischer's exact tests for nominal data and by two-sample *t* tests for continuous data; (ii) multivariate analyses were performed using binary logistic regression with estimation of odds ratios (OR) and 95% confidence intervals (95%CI). The most statistically significant (with $p<0.05$) and clinically relevant variables in the univariate step were included in a multivariate model, and only variables with $p<0.05$ were retained for the final model; (iii) the final model was determined by logistic regression to predict NAS ≥5 and based on three parameters: CK18 fragment levels, ALT and MS. The diagnostic performance of this model was determined by constructing a receiver-operating characteristic (ROC) curve and calculating the area under ROC (AUROC) curve for predicting patients with NAS ≥5. From this curve, the best cut-off value was established for the model score; this value maximized the sum of the sensitivity and specificity to identify patient status. The diagnostic accuracy of the logarithmic transformation of the *Nice Model* (Prob(Y<>group) = 1/(1+Exp(-*Nice Model*)) was then determined. Sensitivity, specificity, positive predictive value, negative predictive value and likelihood ratio were determined for each cutoff value of the NiceModel.

Statistical analysis was then carried out on the validation patient cohort (n=154). The main parameters of both cohorts were compared using Chi^2 test or Fischer's exact tests for nominal data, and by two-sample *t* tests for continuous data. The final model obtained in the training patient cohort was applied to the validation patient cohort. The diagnostic performance of the model was determined by constructing a ROC curve and calculating the AUROC curve for prediction of patients with NAS ≥5.

All statistical analyses were performed using NCSS 2007 software.

Results

Pathological findings and univariate analysis

The distribution of NAS scores among the 310 patients in the training patient cohort is shown in the table 1. Using Kleiner's classification, 39 patients had NAS ≥5. Fibrosis reported using bridging fibrosis grade was absent in 13 patients, mild perivenular and/or periportal fibrosis in 281, septal fibrosis in 15 patients and severe fibrosis in one patient.

Using the univariate analysis (Table 2), patients with NAS ≥5 had higher waist circumference, ALT, γGT, triglyceride levels, blood glucose levels, C peptide, glycosylated hemoglobin, HOMA-IR, ferritin and CK18 fragment levels compared to patients with NAS <5. HDL-cholesterol was lower in the more severe patients. Rates of type 2 diabetes mellitus and MS were also higher in patients with NAS ≥5 (Table 2). Neither age nor body mass index (BMI) were associated with NAS ≥5.

Multivariate analysis

Multivariate analysis included a model combining ALT, γGT, HOMA-IR, CK18 fragment levels, gender and MS in the training patient cohort. ALT (OR=1.04 [95%CI: 1.01-1.07], p=0.002), MS (OR=7.3, [95%CI: 1.8-29.4], p=0.005) and CK18 fragments (OR=1.003, [95%CI: 1.0006-1.005], p=0.044) were the only independent variables when NAS ≥5 was the judgment criterion (Table 3).

CK18 fragments, ALT and IDF metabolic syndrome for the prediction of NAS ≥5

AUROC curves for CK18 fragments, ALT and MS were assessed for the prediction of liver disease (NAS ≥5) in the training patient cohort. The AUROC curves were 0.74, 0.78 and 0.74 for the CK18 fragment levels, ALT and MS respectively for the prediction of NAS ≥5.

Model to predict NAS ≥5 in morbidly obese patients

A new model combining these three values was constructed by logistic regression in the training patient cohort, the "*Nice Model*". The equation of this model was: -5.654 + 3.780E-02*ALT (IU/L) + 2.215E-03*CK18 fragment levels (IU/L) + 1.825*(Presence of MS=1). The AUROC curve for the prediction of NAS ≥5 was 0.88 (Figure 1). The diagnostic accuracy of the logarithmic transformation of the *Nice Model* (Prob(Y<>group) = 1/(1+Exp(-*Nice Model*)) is shown in Table 4. The best cutoff value was 0.14. This value was associated with a sensitivity of 0.84, a specificity of 0.86, a positive predictive value of 0.44 and negative predictive value of 0.98. The prevalence of NAS ≥5 was 0.12.

The distribution of values from the *Nice Model* according to liver histology in the training patient cohort is shown in Figure 2. Box plots were constructed either separating patients into simple steatosis (NAS≤2), borderline group (3≤NAS≤4) or definitive NASH (Figure 2A) or between NAS ≥5 and NAS <5 (Figure 2B). *Nice Model* values were significantly higher in patients with NAS ≥5 compared to borderline ($p<0.00001$) and to simple steatosis groups ($p<0.00001$). *Nice Model* values in the borderline group were also significantly higher than in the simple steatosis group ($p<0.00001$). In the same way, patients with NAS ≥5 had significantly higher *Nice Model* values compared to patients with NAS <5 ($P<0.0001$).

Evaluation of the Nice Model in the validation cohort of morbidly obese patients

The training patient and validation patient cohort were similar in terms of age, gender, BMI, ALT, CK18 fragment levels, prevalence of MS, as shown in Table 5. The number of patients with NAS ≥5 was also similar in the two cohorts (12.6% versus 9%, P=NS). AUROC curves for the prediction of NAS≥5 was 0.83 (Figure 1).

Discussion

We propose a new composite scoring system (*Nice Model*) based on ALT, CK18 fragment levels and the presence of MS to diagnose NAS ≥5 in morbidly obese patients. Indeed, the ALT, CK18 fragment levels or presence of MS separately were moderately predictive of NAS ≥5. The association of these three items in the *Nice Model* displayed an AUROC for NAS ≥5 of 0.88 and 0.83 in the training (n=310) and in the validation (n=154) cohort of morbidly obese patients respectively.

Assessment of CK18 fragment levels has recently been proposed as a marker for NASH in patients exposed to NAFLD. The value of this marker was suggested by a pilot study and was confirmed in a large multicentre study in obese patients.[20, 27] The disadvantage of the assessment of CK18 fragment levels is that it is not performed routinely in daily practice, but it has the advantage that it is a commercial ELISA kit. The CK18 fragment levels have also been studied in morbidly obese patients. In a cohort of 99 morbidly obese patients, CK18 fragment levels were found to be associated with NASH. CK18 fragment levels AUROC curve was 0.88.[21] This value was higher than the one found in our training patient group (AUROC curve value only of 0.74) to predict NAS ≥5. Difference in the diagnostic accuracy could be due to the influence of fibrosis on the CK18 fragments levels. As reported by the previous authors CK18 fragments levels were significantly higher in subjects with moderate to severe fibrosis versus those with no or mild fibrosis.[21]

In order to improve the prediction of definitive NASH, we attempted to develop a new scoring system combining variables significantly associated with NAS ≥5 in univariate and multivariate analysis. These variables were CK18 fragment levels, ALT and the presence of metabolic syndrome. ALT level has previously been related to inflammatory liver damage during NASH in morbidly obese patients and to an elevated risk of appearance of MS, diabetes and cardiovascular events.[17, 28] However, ALT alone would not be sufficient for predicting NASH because it has been reported normal levels of transaminases in patients with proven steatohepatitis and/or significant or severe fibrosis due to NASH.[29-31]

Presence of Metabolic syndrome in our model is not surprising because it is strongly associated with liver complications. However, in our cohort, metabolic syndrome alone is poorly predictive of definitive NASH.

Our scoring system offers the original association of CK18 fragment levels, ALT and MS to diagnose definitive NASH. Several non-invasive tests have been proposed for the diagnosis of NASH. Among the different models proposed, [16, 19, 22, 33, 34] we evaluated only those with items available in our cohort.[15, 17, 18] Respective AUROC of these models applied in the entire cohort (n=464) are summarized in table 6.

One of the weaknesses of this study is that our model is developed among morbidly obese patients. The clinical utility of this model will be fully acquired only when a validation on overweight and simple obese patients will have been carried out. Another point is the relatively small number of patients with NAS≥5 which could limit the statistical power of analyses.

Pure steatosis had classically a relatively good prognosis whereas steatohepatitis was associated with liver fibrosis. However, it was recently suggested that patients with low NAS could develop NASH and fibrosis progression.[32] Even if patients with definitive NASH could have a more severe prognosis, clinicians should keep in mind that all patients with NAFLD should undergo periodic assessment and treatment.

In conclusion, a new composite model, the *Nice Model*, for the diagnosis of definitive NASH in morbidly obese patients based on ALT and CK18 fragment levels and on the presence of a MS is proposed. The diagnostic accuracy of this model was better than using CK18 fragment levels, ALT and the presence of a MS alone. Possibility of the *Nice Model* to be an alternative to liver biopsy in the follow up of obese patients and in the evaluation of treatments aimed at preventing the progression of liver disease in these high risks patients should be assessed. The identification of non invasive scoring system independent of the severity of obesity and the ethnicity constitute an important challenge for the diagnosis of NASH.

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Most important aspect of the paper: A non invasive scoring system including metabolic syndrome, alanine aminotransferase and Cytokeratin-18 fragments was able to accurately predict definitive NASH in morbidly obese subjects.

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Abbreviations:

ALT: alanine aminotransferase

AST: aspartate aminotransferase

AUROC: area under receiver-operating characteristic (curve)

BMI: body mass index

BP: blood pressure

CRP: C-reactive protein

γGT: γ glutamyl transferase

HDL: high-density lipoprotein

HOMA-IR: homeostasis model assessment of insulin resistance

LDL: low-density lipoprotein

MS: metabolic syndrome

NAFLD: non-alcoholic fatty liver disease

NAS: NAFLD activity score

NASH: non-alcoholic steatohepatitis

ROC: receiver-operating characteristic

TABLES

Table 1: Distribution of parameters according to NAS score in the training morbidly obese patient cohort (n=310).

NAS	n	Age (years)	Gender (M/F)	BMI (kg/m²)	CK18 fragment levels (IU/L)	ALT (IU/L)	IDF MS (Absent/Present)
0	20	30.5 ± 9.2	0/20	43.7 ± 2.6	216.3 ± 114.6	21.0 ± 8.5	16/4
1	105	40.2 ± 11.0	8/97	44.7 ± 4.7	231.3 ± 114.6	23.7 ± 10.9	59/46
2	72	40.5 ± 10.0	10/62	44.4 ± 5.4	225.4 ± 138.3	31.8 ± 25.5	35/37
3	59	40.8 ± 11.0	11/48	44.6 ± 4.8	253.4 ± 158.9	43.3 ± 21.1	25/34
4	15	42.4 ± 8.7	5/10	45.5 ± 5.0	254.2 ± 130.6	38.6 ± 20.6	7/8
5	36	41.0 ±10.3	9/27	45.3 ± 6.4	393.5 ± 250.4	63.0 ± 47.8	7/29
6	3	50.6 ± 4.7	0/3	41.9 ± 2.3	935.2 ± 198.2	118.7 ± 28.3	0/3
7	0	-	-	-	-	-	-
8	0	-	-	-	-	-	-

Results are expressed as means ± standard deviation, or as absolute numbers. Metabolic syndrome (MS) was defined according to the definition of the International Diabetes Federation (IDF). NAS: Non-Alcoholic Fatty Liver Disease Activity Score ; M: Male ; F: Female ; BMI: Body Mass Index; ALT: alanine amino-transferase.

Table 2: Univariate analysis of the training patient cohort (n=310) according to the severity of liver disease.

	Liver disease according to NAFLD Activity Score (NAS)		
	NAS <5 (n=271)	NAS ≥5 (n=39)	P
Age (years)	39.8 ± 10.8	41.7 ± 10.2	NS
Gender (M/F)	34/237	9/30	NS
BMI (kg/m²)	44.6 ± 4.8	45.0 ± 6.2	NS
Waist circumference (cm)	119.7 ± 13.1	126.9 ± 15.6	0.002
ALT (IU/L)	30.7 ± 20.1	67.4 ± 48.8	<0.0000001
γGT (IU/L)	38.8 ± 34.2	73.6 ±54.4	<0.0000001
HDL Cholesterol (mmol/L)	1.4 ± 0.3	1.3 ± 0.3	0.008
Triglyceride (mmol/L)	1.65 ± 1.16	2.6 ± 2.1	0.00002
Blood glucose level (mmol/L)	5.9 ± 2.2	7.6 ± 3.3	0.00006
C-peptide (pmol/L)	1047.5 ± 405.2	1402.9 ± 442.8	0.000006
Glycosylated hemoglobin (%)	5.9± 1.1	6.8 ± 1.6	0.000009
HOMAIR	5.5 ± 5.9	9.2 ± 6.6	0.0007
Ferritin (µmol/L)	74.3 ± 77.5	142.8 ± 165.6	0.00003
CK18 fragment levels (IU/L)	234.9 ± 132.0	445.9 ± 292.5	<0.0000001
Type 2 Diabetes (%)	19.6	43.6	0.03
IDF MS (%)	47.6	82.1	0.003

Patients were classified as without or with a NAFLD Activity Score (NAS) <5 or ≥5. Quantitative results are expressed as means ± standard deviation. Metabolic syndrome (MS) was defined according to the definition of the International Diabetes Federation (IDF); M :Male ; F :Female ; NS : Not Significant; BMI: Body Mass Index; ALT: alanine amino-transferase; γGT : Gamma Glutamyl Transpeptidase ; HDL: high density lipoprotein ; HOMA-IR: homeostasis model assessment of insulin resistance.

Table 3: Multivariate analysis of the training patient cohort (n=310) according to the severity of liver disease.

	Liver disease according to NAFLD Activity Score (NAS)		
	NAS ≥5 (n=39) versus NAS <5 (n=271)		
	P	OR	95%CI
ALT	0.002	1.04	1.01-1.07
γGT	0.6	0.99	0.98-1.01
HOMA-IR	0.6	0.98	0.92-1.05
CK18 fragment levels	0.044	1.003	1.00006-1.005
Gender	0.7	0.82	0.21-3.19
IDF MS	0.005	7.3	1.8-29.4

Patients were classified as without or with a NAFLD Activity Score (NAS) <5 or ≥5. Metabolic syndrome (MS) was defined according to the definition of the International Diabetes Federation (IDF); ALT: alanine amino-transferase; γGT: Gamma Glutamyl Transpeptidase; HOMA-IR: homeostasis model assessment of insulin resistance.

Table 4: Diagnostic accuracy of the *NiceModel* to predict NAS ≥5 in the training cohort of morbidly obese patients (n=310).

Cutoff value	Sensitivity	Specificity	Likelihood ratio	Positive Predictive value (prevalence 0.12)	Negative Predictive Value
0.10	0.87	0.80	4.3	0.36	0.98
0.12	0.87	0.81	4.6	0.38	0.98
0.13	0.84	0.84	5.2	0.41	0.98
0.14	***0.84***	***0.86***	***5.9***	***0.44***	***0.98***
0.17	0.68	0.89	6.0	0.44	0.95
0.20	0.58	0.92	6.9	0.47	0.94
0.29	0.48	0.95	10.4	0.57	0.93
0.51	0.35	0.97	14.0	0.65	0.92
0.68	0.23	0.99	26.6	0.78	0.91
0.83	0.16	0.99	38.1	0.9	0.91

Table 5: Comparative analysis between the training (n=310) and the validation (n=154) cohort of morbidly obese patients.

	Training patient cohort (n=310)	Validation patient cohort (n=154)	P
Age (years)	40.0±10.7	39.3±10.8	NS
Gender (M/F)	43/267	19/135	NS
BMI (kg/m²)	44.6±5.0	44.4±5.7	NS
ALT (IU/L)	35.3±28.1	34.4±26.3	NS
CK18 fragment levels (IU/L)	259.2±172.0	242.5±178.0	NS
IDF MS	52% (161/310)	52% (80/154)	NS

Metabolic syndrome (MS) was defined according to the definition of the International Diabetes Federation (IDF); NS : Not Significant; M :Male ; F :Female ; BMI: Body Mass Index; ALT: alanine amino-transferase.

Table 6: AUROC curves of published scoring systems applied in the entire cohort (n=464) to diagnose NAS≥5.

Scoring system	AUROC curve in the original study	AUROC curve in the entire cohort (n=464)
Dixon et al. (HAIR)[17]	0.9	0.77
Gholam et al.[18]	0.82	0.79
Palekar et al.[15]	0.76	0.44

AUROC: area under receiver-operating characteristic. Among published scoring models only those with items available in the cohort were studied.

Figure Legends

Figure 1: *NiceModel* receiver-operating characteristic (ROC) curves for the diagnosis of definitive NASH (NAS ≥5) in morbidly obese patients.
NiceModel ROC curves are shown for the training patient cohort (n=310) and validation patient cohort (n=154).

Figure 2: Distribution of values for the *NiceModel* (based on ALT, CK18 fragment levels and the presence of IDF metabolic syndrome) according to the severity of liver histology in the training cohort (n=310).
(A) Distribution according to NAS values: simple steatosis (NAS ≤2), borderline Group (3≤NAS≤4), and definitive NASH (NAS ≥5). (B) Distribution according to NAS values: NAS ≥5 or NAS <5.

STARD checklist for reporting of studies of diagnostic accuracy
(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	Page 1
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	Page 4
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	Page 5
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	Recruitment was based on systematic enrolment during bariatric surgery
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	All consecutive morbidly obese patients were analysed. The cohort was arbitrally split in a training and a validation cohort.
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	It is a prospective study.
Test methods	7	The reference standard and its rationale.	Systematic liver biopsy which is the Gold Standard for NASH
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	Yes, page 5 and 6
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	"Definitive NASH" (NALFD Activity Score ≥5) was retained according to Kleiner's classification.
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	All liver biopsies were read by a specialized pathologist (MCSP). Kleiner's classification was secondly determined by MCSP and SP.
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	Yes, the readers of the liver biopsy were blind to the results of the proposed model.

Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	Comparisons were done using the Chi^2 test or Fischer's exact tests for nominal data and by two-sample *t* tests for continuous data. Multivariate analyses were performed using binary logistic regression with estimation of odds ratios (OR) and 95% confidence intervals (95%CI). Only variables with $p<0.05$ in multivariate analyses were retained for the final model. The diagnostic performance of this model was determined by constructing a receiver-operating characteristic (ROC) curve and calculating the area under ROC (AUROC) curve for predicting patients with NAS ≥5.
	13	Methods for calculating test reproducibility, if done.	Not done.
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	Page 5
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	Table 1 and table 5
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	All the patients satisfying the criteria for inclusion were enrolled in the study.
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	Clinical and biological data were collected few days before the bariatric sugery after overnight fasting. Liver biopsy was done during surgery.
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	Among morbidly obese patients enrolled in the training cohort 12.6% had a "definitive NASH" (NALFD Activity Score ≥5)
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	The distribution of the test results by the results of liver biopsy is presented in Figure 2.
	20	Any adverse events from performing the index tests or the reference standard.	No adverse events occurred.
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	AUROC curves of the scoring system were 0.88 and 0.83 in the training and in the validation group respectively.
	22	How indeterminate results, missing data and outliers of the index tests were handled.	A borderline group is determined in the Kleiner's classification. Diagnostic accuracy is given in the table 4.
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	AUROC curves of the scoring system were 0.88 and 0.83 in the training and in the validation group respectively.
	24	Estimates of test reproducibility, if done.	Not done

DISCUSSION	25	Discuss the clinical applicability of the study findings.	ALT level and Metabolic syndrome determination are done routinely. Blood levels of apoptotic caspase-3 generated CK18 fragment levels were measured using a commercial kit. External validation in non morbidly obese patients will be necessary. Pages 10 & 11.

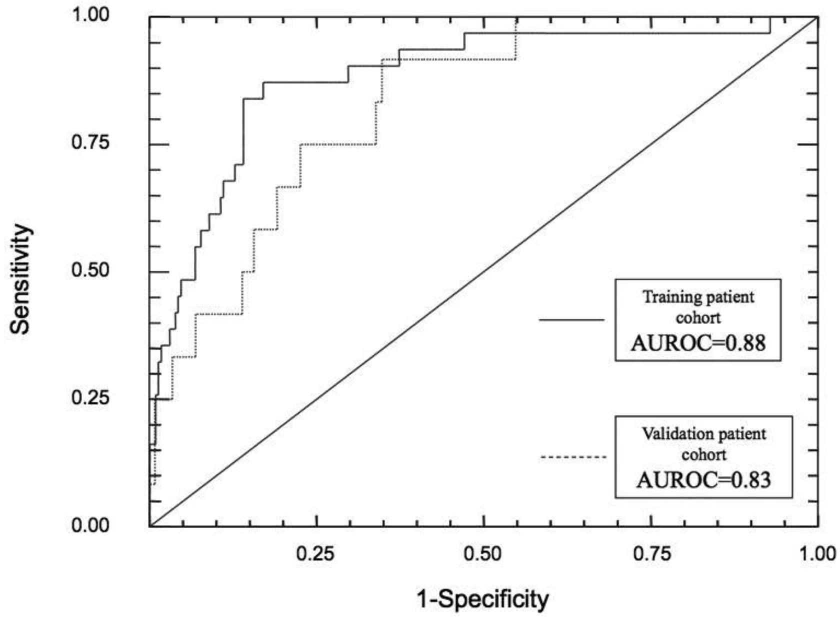

Figure 1: NiceModel receiver-operating characteristic (ROC) curves for the diagnosis of definitive NASH (NAS ≥5) in morbidly obese patients.
NiceModel ROC curves are shown for the training patient cohort (n=310) and validation patient cohort (n=154).

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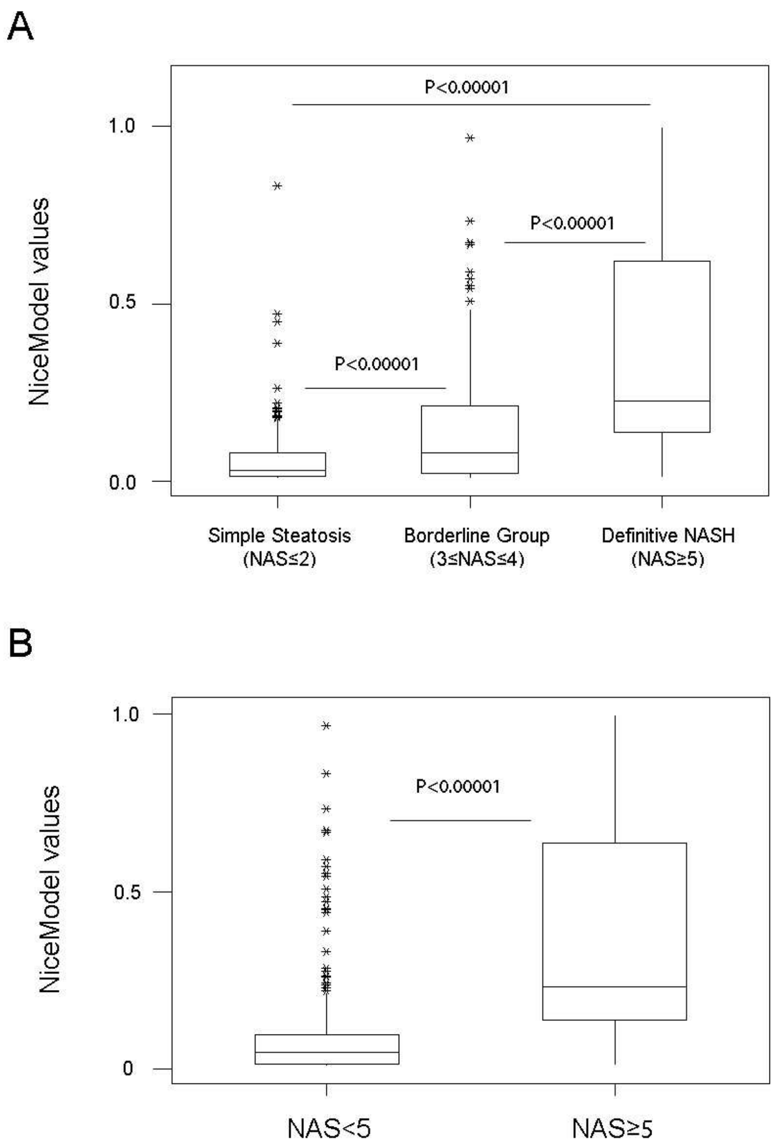

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230x324mm (96 x 96 DPI)